The significance of Ser^{1029} of the heat-stable enterotoxin receptor (STaR): relation of STa-mediated guanylyl cyclase activation and signaling by phorbol myristate acetate

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Abstract To characterize Ser^{1029} in STaR at a consensus sequence of phosphorylation site by PKC, two mutants of mS1029A with replacement of Ser^{1029} to Ala^{1029} and CΔ1029 lacking 22 amino acids including Ser^{1029} were prepared. Preincubation of the wild type-STaR (wt-STaR) transfectant with 1 μM PMA caused additional STa-mediated guanylyl cyclase (GC) activation compared to control, whereas the mS1029A- and CΔ1029-transfected cells did not show a similar enhanced GC activation by PMA. After metabolic labeling with [^{32}P]phosphate, transfected cells with wt-STaR and mutants were incubated with 1 μM PMA. Subsequent ^{32}P-radiolabeled proteins were immunoprecipitated using anti-STaR antibody, and analyzed by autoradiography after separation on SDS-PAGE. The immunoprecipitated wt-STaR but not mS1029A and CΔ1029 had a significant radioactivity. These results suggest that the effect of PMA on wt-STaR transfectants may be caused by phosphorylation of Ser^{1029}. The CΔ1012 mutant, with further truncation (Gln^{1012}–Phe^{1050}) of the carboxy terminus, did not show STa-mediated GC activation. Based on these data, these 17 amino acids (Gln^{1012}–Ala^{1028}), essential for signaling of GC activation by STa, have an abundance of basic amino acids which might be functionally influenced by phosphorylation of Ser^{1029}.

Key words: Heat-stable enterotoxin receptor; Guanylyl cyclase; Phorbol myristate acetate

1. Introduction

The heat-stable enterotoxin (STa) of *Escherichia coli* is an 18 or 19 amino acid peptide toxin (STp or STh) that causes diarrhea in humans and domestic animals [1,2]. STa binds to a cell surface receptor (STaR/GC-C) [3–6] in the intestine, which subsequently leads to activation of a guanylyl cyclase (GC) catalytic domain resulting in an accumulation of intracellular cyclic GMP (cGMP). The increase in cGMP causes fluid secretion, in part, through the phosphorylation of cystic fibrosis-related chloride channels possibly by activation of protein kinase A [7]. STaR has common structural features among membrane-associated GCs, including an extracellular ligand-binding domain, a single transmembrane domain, a kinase-homology domain and a GC catalytic domain. In a prolonged carboxy-terminal tail adjacent to the GC catalytic domain, STaR has an extra 63 or 62 amino acids compared to the natriuretic peptide receptors, NPR-A/GC-A or NPR-B/GC-B (Fig. 1).

In the T84 colonic carcinoma intestinal cell line, phorbol esters have an additive effect on STa-stimulated GC activity consistent with a possible role for phosphorylation by protein kinase C (PKC) [8]. It is notable that this activation effect of phorbol myristate acetate (PMA) on STa-stimulated GC activity of STaR is opposite to the inhibitory effects of PMA on NPR-A-stimulated GC activity [9–12]. More recently, Crane et al. have demonstrated that phosphorylation of STaR is blocked by addition of a synthetic peptide, KPRRVASYKKG, corresponding to the sequence around Ser^{1029} in the carboxy-terminal tail of the STaR, suggesting the putative phosphorylation site to be Ser^{1029} (personal communication). Here, we report the significance of phosphorylation of Ser^{1029} of STaR in mediating signaling by PMA by using various mutants of pig STaR. We describe that a CΔ1012 mutant, which has the GC catalytic domain and lacks 39 amino acids in the C-terminus of STaR, loses its inherent properties to be activated by STa.

2. Materials and methods

2.1. StaR mutant constructs

The pCG-STaR which contains the full-length pig STaR cDNA [6] under the cytomegalovirus promoter [13] was used for this study as wild-type STaR (wt-STaR). A STaR point mutant, mS1029A was constructed using an oligonucleotide (5′-CA ACC CGG GTA GCC GCA TAC AAG AAA GGC A-3′) according to Kunkel [14]. STaR deletion mutant cDNAs were constructed by the use of polymerase chain reaction.

2.2. Intracellular cGMP determination

293T cells are a human embryonic kidney cell line infected with adenovirus carrying an SV40-large T-antigen expression plasmid [15]. The cells were grown in DMEM medium supplemented with 10% fetal calf serum. The cells were transfected with 10 μg of the pCG mammalian expression vector in which we inserted the pig STaR gene (pCG-STaR) or its mutated gene as reported in [6]. Transfection was accomplished using the calcium phosphate method [16].

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Abbreviations: STa, heat-stable enterotoxin; STh, heat-stable enterotoxin produced by a human strain of *Escherichia coli*; STaR, heat-stable enterotoxin receptor; GC, guanylyl cyclase; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium.

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                    985   990          1000         1010         1020         1030         1040         1050
STaR     :···········YNLPTPPTAENQQRLQAEFVDMIASSLQKRQASGIKNRKPTRVASYKKGTLEYLQLNTTDNESTHF

CΔ988    :···········YNL
CΔ996    :···········YNLPTPPTAEN
CΔ1012   :···········YNLPTPPTAENQQRLQAEFVDMIASSL
CΔ1029   :···········YNLPTPPTAENQQRLQAEFVDMIASSLQKRQASGIKNRKPTRVA
                                                 ●
mS1029A  :···········YNLPTPPTAENQQRLQAEFVDMIASSLQKRQASGIKNRKPTRVAAYKKGTLEYLQLNTTDNESTHF
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Fig. 1. STaR mutant constructs in carboxy-terminal regions.

cGMP assays were done as described previously [6] using a radioimmunoassay kit (Yamasa cGMP assay kit, Yamasa, Japan).

2.3. Preparation of anti-STaR and immunoprecipitation of recombinant STaR

A synthetic peptide, CTNHVSLKIDDDRRRD (amino acids 461–475 with an additional amino-terminal Cys residue), representing the begining of the kinase-homology domain of pig STaR [6] was synthesized and coupled to bovine serum albumin. Rabbits were immunized subcutaneously with the antigen. The antiserum with the highest titer in enzyme-linked immunosorbent assay, GCINT1, was used in this study after determination of its specificity against recombinant STaR using Western blot analysis. Metabolic labeling and immunoprecipitaion of STaR and its mutants with [^{35}S]methionine, [^{35}S]cysteine, and [^{32}P]phosphate were performed according to Vaandrager et al. [17]. The STa used here is a synthesized STh reported in [18]. Other reagents were of analytical grade.

3. Results and discussion

To characterize the role of Ser^{1029} in STaR, two mutated genes encoding a mS1029A with substitution at Ser^{1029} to Ala^{1029} and CΔ1029 deleted with Ser^{1029} to the carboxy-terminal amino acid Phe^{1050} were constructed (Fig. 1). When the mS1029A- and CΔ1029-transfected cells were incubated with 1 μM STa for 10 min, the intracellular cGMP concentration of the transfected cells increased in a manner similar to wt-STaR transfectants (Fig. 2). Pretreatment of the wt-STaR transfectants with 1 μM PMA for 10 min prior to the addition of STa in the culture medium provoked a more potent stimulatory effect of STa on GC activity of wt-STaR (Fig. 2). This is similar to the results previously shown by Weikel et al. [8]. However, this additive effect of PMA was not observed in the mS1029- and CΔ1029-transfected cells (Fig. 2). These results suggest that Ser^{1029} in the carboxy-terminal tail of wt-STaR may be a phosphorylation site by PKC resulting from treatment with PMA, and that the phosphorylated Ser^{1029} induced a suitable conformational change in the GC catalytic domain which in turn led to the more active form.

We investigated further the possible phosphorylation of Ser^{1029} addressing the suspicion that mS1029A and CΔ1029 were not expressed on the transfected cells by metabolic labeling with [^{35}S]methionine and [^{35}S]cysteine. Fig. 3A shows that mS1029A and CΔ1029 mutants were expressed in the transfected cells at a rate similar to those of wt-STaR and three carboxy-terminal tail deletion mutants (CΔ988, CΔ996, and CΔ1012, Fig. 1) by imunoprecipitation analysis after metabolic labeling proteins with [^{35}S]methionine and [^{35}S]cysteine. After preincubation of the wt-STaR transfectants and mutants with [^{32}P]phosphoric acid for 3 h and subsequent treatment with 1 μM PMA for 1 h, the ^{32}P-labeled STaR and the

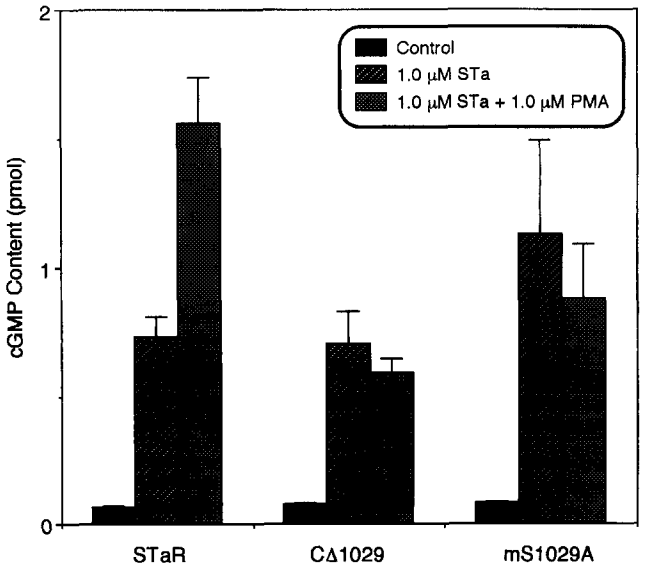

Fig. 2. Effect of PMA on GC activity in STaR or its mutants expressing 293T cells. STaR expressed 293T cells were treated with or without PMA for 10 min at 37°C, and then were treated with 1 μM STa.

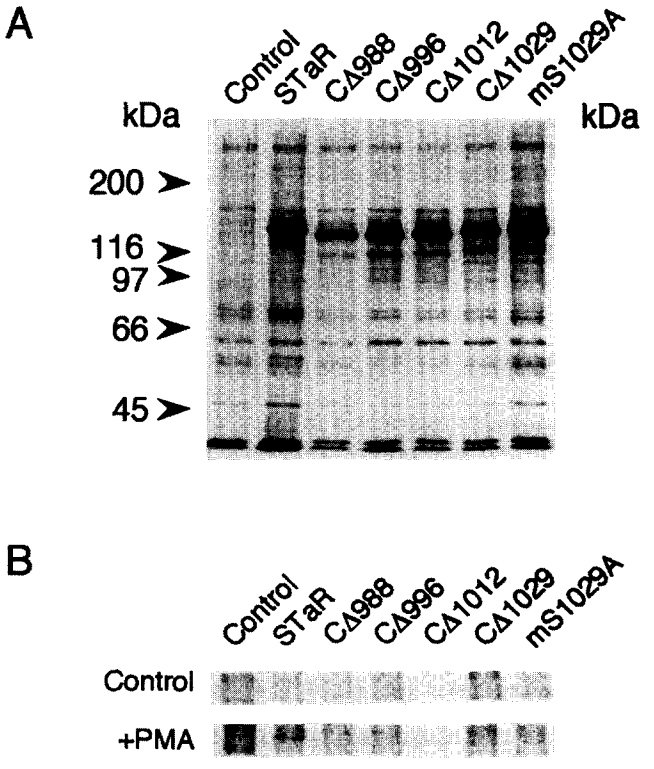

Fig. 3. Metabolic labeling of STaR or its mutants. (A) Immunoprecipitations of ^{35}S-labeled STaR or its mutants using anti-STaR antibody, GCINT1. (B) Immunoprecipitations of ^{32}P-labeled STaR or its mutants incubated with or without PMA for 1 h.

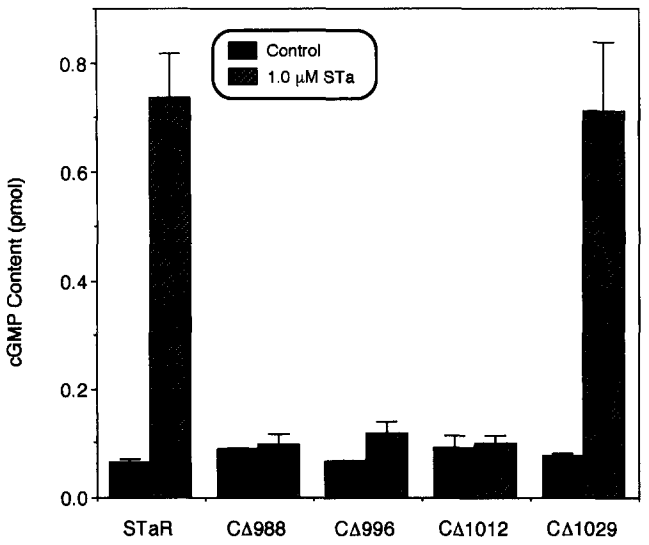

Fig. 4. cGMP production in response to STh(1–19) by STaR or its mutants. 293T cells transfected with the indicated genes (10 μg) were treated with or without 1 μM STa for 10 min at 37°C. Results are expressed as the mean ± S.E.M. of four determinations.

mutants were immunoprecipitated using anti-STaR antibody, GCINT1 (Fig. 3B). No significant radioactive incorporation into the two mutants mS1029A and CΔ1029 was observed, even though wt-STaR was detected as a radioactive band on the SDS-PAGE gel. Three carboxy-terminal deletion mutants lacking Ser^{1029} also did not incorporate ^{32}P radioactivity. This result strongly suggests Ser^{1029} is a target site of phosphorylation by PKC because the two mutants mS1029A and CΔ1029 lacked Ser^{1029} by amino acid substitution or deletion, respectively. This result is also consistent with the presence of a PKC consensus phosphorylation sequence (RXXSXK) surrounding Ser^{1029} (RVASYK) [19].

To understand the importance of the carboxy terminus in the STa-mediated reaction of GC and in the modulation of the effects of STa by PKC, further examination compares the effects of other deletions of the carboxy-terminal tail on STa-induced GC-activation using CΔ988, CΔ996, CΔ1012, and CΔ1029 (Fig. 1). When the CΔ1029 transfected cells were incubated with 1 μM STa for 10 min, intracellular cGMP concentration increased at a similar level to that in wt-STaR as shown in Fig. 4. However, no increase in STa-mediated intracellular cGMP was observed in the cells transfected with CΔ988, CΔ996, and CΔ1012. As shown in Fig. 3A, CΔ988, CΔ996, and CΔ1012 were expressed at similar levels to wt-STaR in 293T cells as judged by immunoprecipitation. All mutant constructs showed similar levels of cell surface expression as wt-STaR by immunofluorescence staining (data not shown). These results suggest that 22 amino acid residues (Ser^{1029}–Phe^{1050}) including Ser^{1029} at the carboxy-terminal tail of STaR are not required for STa-mediated GC activation. STa-mediated activation of the GC domain occurs without phosphorylation of Ser^{1029}. However, further activation of STaR by pretreatment with PMA before exposure to STa might be due to phosphorylation of Ser^{1029}. The sequence consisting of 17 amino acids at positions 1012–1028 adjacent to Ser^{1029} has an abundance of basic amino acids such as Arg or Lys residues. This sequence may functionally affect the ability of STa to activate the GC catalytic domain. The mutant CΔ1012 lacking these 17 amino acids at 1012–1028 lost its inherent property of STa-mediated GC activation even though CΔ1012 could bind to STa, indicating that this deletion mutation inactivated catalytic function. Phosphorylation of Ser^{1029} may introduce an ionic interaction between the phosphoric acid and these basic amino acids, which in turn allows it to be in a more reactive state and generate an exaggerated response to STa. These data support the schema proposed by Weikel et al. [8]. Rudner et al. [20] recently proposed that the binding of STa to STaR removes the inhibitory effects of the kinase-homology domain and promotes an interaction between GC catalytic domains. It is therefore likely that the 17 amino acids at 1012–1028 play a role in the interaction of GC catalytic domain or the kinase-homology domain. More extensive analysis of these 17 amino acids is presently under way.

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